Scaffold fabrication in a perfusion culture microchamber array chip by O_2 plasma bonding of poly(dimethylsiloxane) protected by a physical mask

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Extracellular matrix (ECM) proteins are required for cell culture. In this paper, we report the use of O_2 plasma bonding to fabricate a perfusion culture microchamber array chip with identical-size ECM spots in the isolated microchambers. The chip was fabricated by assembly of two poly(dimethylsiloxane) (PDMS) layers, a microfluidic network layer, and an ECM array layer, which were aligned and then bonded by O_2 plasma oxidation with protection of the ECM microarray with a physical mask made from PDMS. We successfully cultivated Chinese hamster ovary K1 cells in the microchambers with fibronectin. In the fibronectin microchambers, the cells adhered and extended after 12 h of static culture and then grew over the course of 1 d of perfusion culture. © *2011 American Institute of Physics.* [doi:10.1063/1.3576933]

I. INTRODUCTION

Microfluidic cell culture chips are expected to be useful for rapid, reproducible assays of small-volume samples without the need for labor-intensive laboratory routine works, e.g., micropipetting.[1,2] We and other researchers have used perfusion culture microchamber array chips for parallel on-chip cell-based assays.[3–8] Most of the previously described microfluidic cell culture chips have been made of poly(dimethylsiloxane) (PDMS), which is a flexible, durable, transparent, and inexpensive polymer from which microstructures can be easily molded by soft lithography.[9–11] Despite the advantages of PDMS, only a limited number of cell types are culturable on PDMS surfaces in the absence of extracellular matrices (ECMs) as scaffolds. Therefore, methods for fabrication of PDMS microchambers modified with appropriate scaffolds are needed for cultivation of target cells on microchips.

ECM microarrays are useful for simultaneous observation and analysis of cell behavior on various ECMs.[12–14] ECMs are spotted on the surface of a slide glass or cover slide, and cells are then cultured on the ECM microarray immersed in a culture dish. However, because the cells are cultured in a continuous liquid phase (the culture medium), contamination of any given ECM spot with soluble factors secreted by the cells on the other ECM spots is a problem. Cellular kinetics generally depend on the combined extracellular stimuli derived from the surface scaffolds and from the soluble factors in the culture medium.[13,15] In contrast, because the individual microchambers of a perfusion culture microchamber array are isolated from one another, not only simultaneous cultivation of different types of cells but also parallel analysis of the cellular responses with various ECMs can be accomplished without cross-talk due to transport of soluble factors between neighboring ECM spots.

Various methods have been used to produce hydrophilic PDMS surfaces,[16–18] including O_2

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plasma oxidation,[9] UV/ozone oxidation,[19] silanization,[20–22] radiation-induced graft polymerization,[23,24] and photoinduced graft polymerization.[25,26] However, the number of applicable methods for fabrication of chips with ECM spots on a PDMS surface inside microchambers is limited.

In this paper, we report the fabrication of a perfusion culture microchamber array chip with isolated ECM spots (diameter: 1.43 mm) of collagen and fibronectin on the bottoms of the individual microchambers. We fabricated the chip by assembling two PDMS layers, a microfluidic network layer and an ECM array layer, which had precisely aligned microarrays of microchambers and ECM spots, respectively. The ECM array layer was fabricated by micropatterned UV-induced graft polymerization and a dehydration-condensation reaction.[27] The ECM array was then protected with a physical PDMS mask, subjected to O_2 plasma oxidation, and then aligned with and bonded to the microfluidic network layer. We used the perfusion culture microchamber array chip with ECMs to cultivate Chinese hamster ovary K1 (CHO-K1) cells.

II. MATERIALS AND METHODS

A. Materials and reagents

SU-8 negative photoresists were obtained from MicroChem (products 2002, 2050, 2075, Newton, MA, USA). PDMS prepolymer and curing agent were obtained from Dow Corning (Sylgard 184, Midland, MI, USA). Tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane was obtained from Gelest (Morrisville, PA, USA). Collagen type I from calf skin (MW: 300 kDa), fibronectin from bovine plasma (MW: 450 kDa), nutrient mixture F-12 HAM, and Dulbecco's phosphate buffered saline solution (*p*H 7.1–7.5) were all obtained from Sigma-Aldrich (St. Louis, MO, USA) and used as received. All other reagents were obtained from Wako Pure Chemical (Osaka, Japan). All aqueous solutions were prepared with water purified with a Milli-Q Water System (Millipore, Billerica, MA, USA).

B. Design of the perfusion culture microchamber array

The perfusion culture microchamber array chip had an 8×8 array of microchambers, and two types of ECMs were immobilized on the bottom of the microchambers in two sets of two columns each [Fig. 1(a)]. The design of the perfusion culture microchamber array is described in a previous report.[8] Cell suspensions are loaded into all 64 microchambers thorough five cell-inlet/medium-outlet main channels [Fig. 1(a), right], and the culture media are supplied thorough four medium-inlet main channels after cell loading [Fig. 1(a), left]. Figure 1(b) is a detail showing the structure of a cell culture microchamber and two microchannels connecting the medium-inlet main channel and the cell-inlet/medium-outlet main channel. The connecting channels are the medium-inlet branch channel and a cell-inlet/medium-outlet branch channel. The medium-inlet branch channel is much narrower than the other microchannels, and the perfusion rate of the microchamber is therefore determined by the largest fluidic resistance of the medium-inlet branch channel in the microfluidic network. The narrow medium-inlet branch channel also works as a passive hydrophobic valve for introduction of a predetermined volume of cell suspension during the cell-loading process, and thus the same number of the cells can be loaded into each microchamber.

C. Microfabrication

To fabricate the perfusion culture microchamber array chip, we prepared three PDMS layers: an ECM array layer, a microfluidic network layer, and a physical mask. All the PDMS layers were designed based on the 8×8 array format.

1. ECM array layer

The ECM array layer (spot diameter: 1.43 mm, pitch: 2.25 mm) was composed of collagen arrays and fibronectin microarrays in two sets of two columns. A cross + was marked on the center of each side for alignment of the physical mask and the microfluidic network layer [Fig. 2(a)]. The

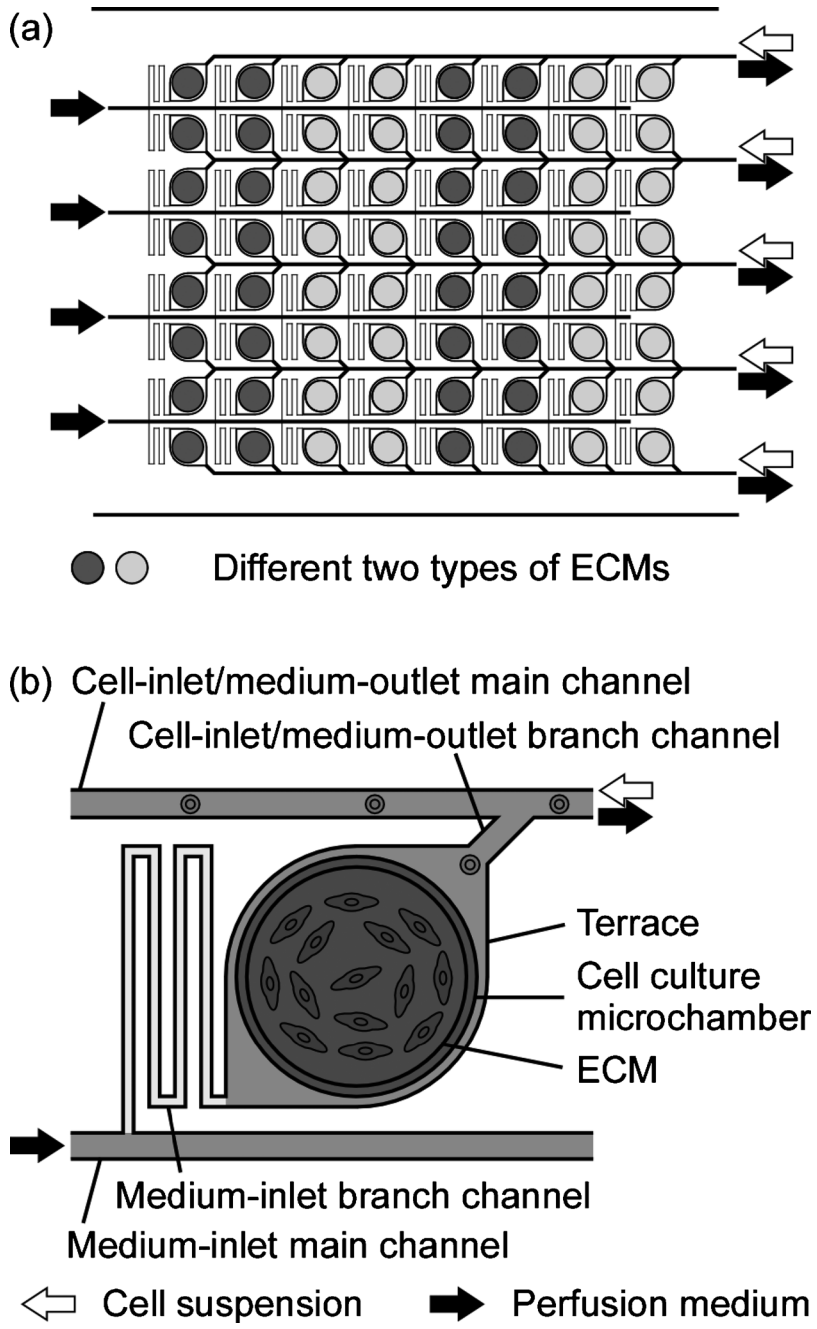

FIG. 1. Structure of the perfusion culture microchamber array chip. (a) Overview of the microfluidic network on the chip. (b) Detail of a microchamber: microchamber (dark gray; depth: 271.2 μm, diameter: 1530 μm); microchannels and terrace (gray; depth: 52.5 μm, width: 100 μm); and microchannel (light gray; depth: 4.5 μm, width, 40 μm).

fabrication of the ECM array layer by micropatterned UV-induced graft polymerization and micropatterned dehydration-condensation reaction was described in a previous report.[27] Briefly, a microarray of poly(acrylic acid) (PAA) as a base polymer for ECM immobilization was grafted on a PDMS plate (width: 25.0 mm, length: 76.0 mm, thickness: 1 mm) by micropatterned UV-induced graft polymerization through a photomask with a transparent pattern corresponding to the 8×8 array.[28] Collagen and fibronectin were subsequently immobilized on the PAA microarray by

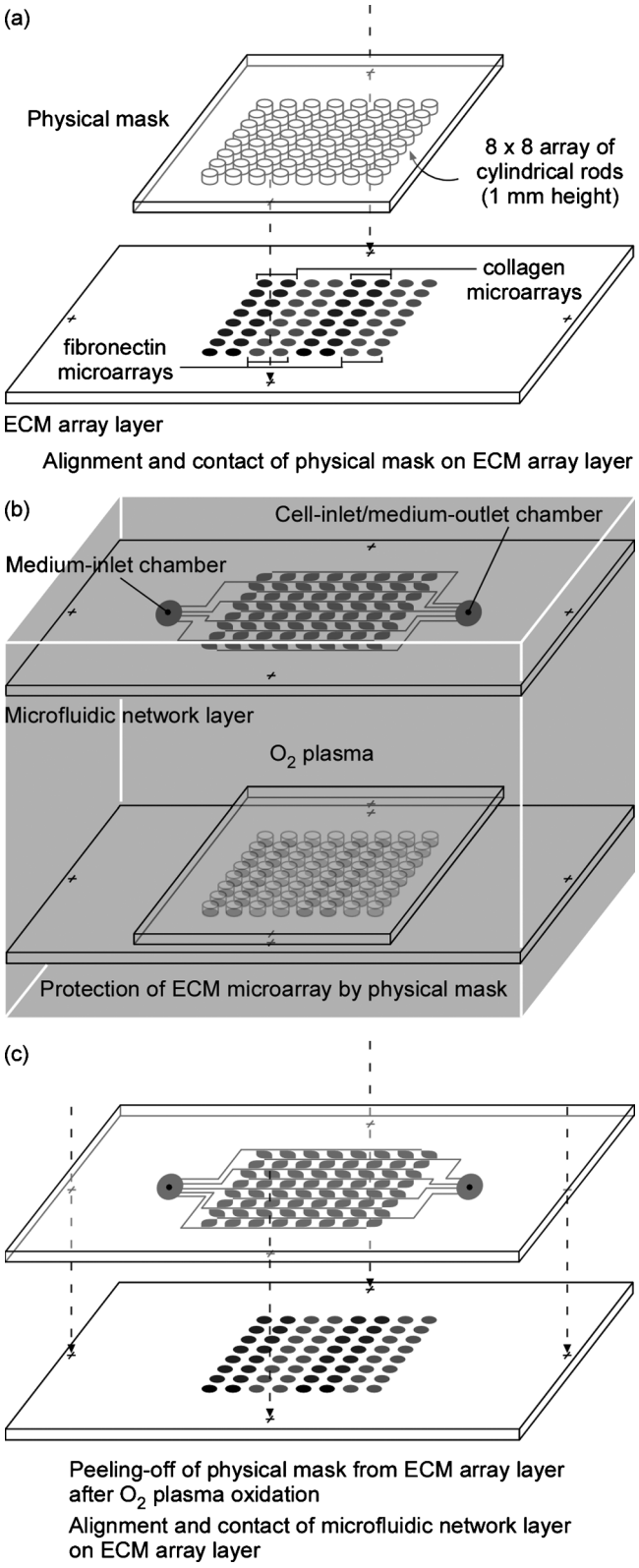

FIG. 2. Fabrication of the perfusion culture microchamber array chip. (a) Masking of the ECM microarray. (b) Oxidation of the adhered surfaces with O_2 plasma for bonding. (c) Bonding of the microfluidic network layer and the ECM array layer.

a dehydration-condensation reaction through a microfabricated stencil with through-holes corresponding to the 8×8 array. This procedure resulted in identical spots of collagen and fibronectin in an arrangement that precisely matched the 8×8 array.

2. Physical mask

The physical mask (width: 25.0 mm, length: 38.0 mm, thickness: 1 mm) had an 8×8 array of cylindrical rods (diameter: 1.53 mm, height: 1 mm, pitch: 2.25 mm) and + marks for alignment with the ECM array layer [Fig. 2(a)]. The physical mask protected the ECM array during the O_2 plasma oxidation process used to bond the microfluidic network layer and the ECM array layer. The physical mask was fabricated by photolithography and replica molding of PDMS.[9,11] A master template with an array of small pits (diameter: 1.53 mm, depth: 1 mm, pitch: 2.25 mm) was created by photolithography with an SU-8 negative photoresist (SU-8 2075) and a photomask with a light-blocking pattern corresponding to the 8×8 array. The photoresist was spin-coated on the silicon wafer (thickness: 250 μm), and the wafer was soft-baked. The spin-coating and soft-baking process was repeated four times to form the 1-mm-thick photoresist layer. The photoresist layer was irradiated with UV light through the photomask and postbaked. After postbaking, the photoresist pattern was developed in ethyl lactate and washed with isopropanol. After being washed, the master template was treated with tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane at 25 °C for 3 h. PDMS prepolymer and curing agent were thoroughly mixed at a 10:1 ratio and poured onto the master template. After curing in an oven at 120 °C for 2 h, the micropatterned PDMS plate was peeled from the master template.

3. Microfluidic network layer

The microfluidic network layer has a medium-inlet chamber, an 8×8 array of microchambers (diameter: 1.53 mm, depth: 250 μm, pitch: 2.25 mm), a terrace structure, a cell-inlet/medium-outlet chamber, connecting microchannels of different depths, and marks for alignment with the ECM array layer [Fig. 2(b)]. A master template with a multithickness pattern for the microchambers and microchannels was created by multilayer photolithography[29] with modifications.[8] SU-8 negative photoresists (SU-8 2002, 2050, and 2075) and photomasks for each layer pattern were used. A sequence of four steps (spin-coating, soft-baking, exposure, and postexposure baking) was repeated for three cycles to fabricate the multithickness photoresist pattern. In the first cycle, a photoresist layer of SU-8 2002 was created by spin-coating, and the pattern for the medium-inlet branch channel (depth: ~5 μm) was created. In the second cycle, the prior photoresist layer was spin-coated with SU-8 2050, and the patterns for the medium-inlet main channel, the cell-inlet/medium-outlet main channel, the cell-inlet/medium-outlet branch channel, and the terrace structure were created (depth: ~50 μm). In the final cycle, the prior two photoresist layers were spin-coated with SU-8 2075, and the patterns for the medium-inlet chamber, the cell-inlet/medium-outlet chamber, and the cell culture microchambers were created (depth: ~250 μm). The development of the photoresist patterns and the replica molding of the PDMS were carried out as described in Sec. II C 2.

4. Fabrication of the perfusion culture microchamber array chip

We fabricated the perfusion culture microchamber array chip by bonding the microfluidic network layer, the ECM array layer, and two macroscopic liquid reservoirs for the medium and the cell suspension. PDMS layers are often bonded by O_2 plasma oxidation of the PDMS surface.[9] Because ECM proteins can be damaged by O_2 plasma, we protected them from the plasma by using the physical mask. The 8×8 array of cylindrical rods on the physical mask was aligned and placed in contact with the 8×8 array of ECMs on the ECM array layer [Fig. 2(a)], and then the adhered surface of the microfluidic network layer and the masked ECM array layer were exposed to O_2 plasma in a plasma reactor (PR500, Yamato Scientific Co., Tokyo, Japan) [Fig. 2(b)]. After oxidation, the physical mask was peeled from the ECM array layer. The oxidized PDMS surfaces of the microfluidic network layer and the ECM array layer were placed in tight contact with each

other after alignment [Fig. 2(c)]. Thus, an ECM spot was situated on the bottom of the corresponding microchamber, and the microfluidic network was simultaneously enclosed. The two macroscopic liquid reservoirs were fabricated from PDMS with an acrylic resin template, and the reservoirs were bonded to the medium-inlet chamber and the cell-inlet/medium-outlet chamber.

D. Evaluation of the protective effect of the physical mask

We evaluated the protective effect of the physical mask during the bonding process. Tetramethylrhodamine isothiocyanate (TRITC)-labeled fibronectin was homogeneously immobilized over the entire homogeneous PAA-grafted PDMS surface by means of UV-induced graft polymerization and a hydration-condensation reaction. The physical mask was placed on the fibronectin-coated PDMS surface, and the surface was exposed to O_2 plasma. The surfaces of the fibronectin-coated PDMS before and after the O_2 plasma oxidation were observed with a fluorescence imaging device consisting of a fluorescence filter block (Olympus, Tokyo, Japan), a CCD color digital camera module (DFW-SX910, Sony Corp., Tokyo, Japan), and a light source (Lightningcure LC6, Hamamatsu Photonics Co., Shizuoka, Japan). Fluorescence images were recorded with commercial VISION FREEZER VFS-42 software (version 3.0, Chori Imaging Corp., Kanagawa, Japan) with an exposure time of 1 s.

E. Seal test of fabricated perfusion culture microchamber array chip

We evaluated the seal between the microfluidic network layer and the ECM array layer by the bonding process with O_2 plasma oxidation. To visualize sealed microfluidic networks of the perfusion culture microchamber array chip, a solution of 5 wt % gardenia blue was added to the medium-inlet chamber using a micropipette and the solution was introduced into the microfluidic network by applying 8 kPa of pressure to the medium-inlet chamber. The pressure was applied with an S100 air pump (Atem Corp., Tokyo, Japan) and was controlled with a PR-4102 pressure regulator (GL Science, Tokyo, Japan) and a handheld manometer (PG-100, Nidec Copal Electronics Corp., Tokyo, Japan).

F. Cell culture in the perfusion culture microchamber array chip

CHO-K1 cells obtained from the Riken Bioresource Center (Tsukuba, Ibaraki, Japan) were maintained in a nutrient mixture consisting of F-12 HAM medium supplemented with 10% fetal bovine serum (Gibco BRL, Rockville, MD, USA), penicillin/streptomycin, and nonessential amino acids at 37 °C in a humidified atmosphere containing 5% CO_2. After the addition of trypsin, the CHO-K1 cells were harvested and then suspended in the culture medium. Before loading of the cells, the perfusion culture microchamber array chip was sterilized by irradiation for 1 h with UV light from a germicidal lamp on a clean bench. The cell suspension (3.5×10^5 cell/mL) was added to the cell-inlet/medium-outlet chamber with a micropipette, and the cells were loaded into the microchambers by application of 15 kPa of pressure to the cell-inlet/medium-outlet chamber through a sterile air-vent filter. The cell-loaded microchip was incubated under static culture conditions to induce cell adherence to the ECM spots on the bottoms of the microchambers. After 12 h, culture medium was added to the medium-inlet chamber, and continuous perfusion culture was carried out for 1 d by application of 8 kPa of pressure to the medium-inlet chamber in a CO_2 incubator. The cultured CHO-K1 cells were observed with a phase-contrast microscope (IX71, Olympus Corp., Tokyo, Japan) equipped with a cooled CCD camera (VB7010, Keyence, Osaka, Japan).

III. RESULTS AND DISCUSSION

A. Protection of the ECM microarray by the physical mask

We evaluated the protective effect of the physical mask by analyzing the fluorescence images of the homogeneous fibronectin-coated PDMS surface before O_2 plasma oxidation and the protected surface after irradiatoxidation [Figs. 3(a) and 3(b), respectively]. The TRITC-labeled fi-

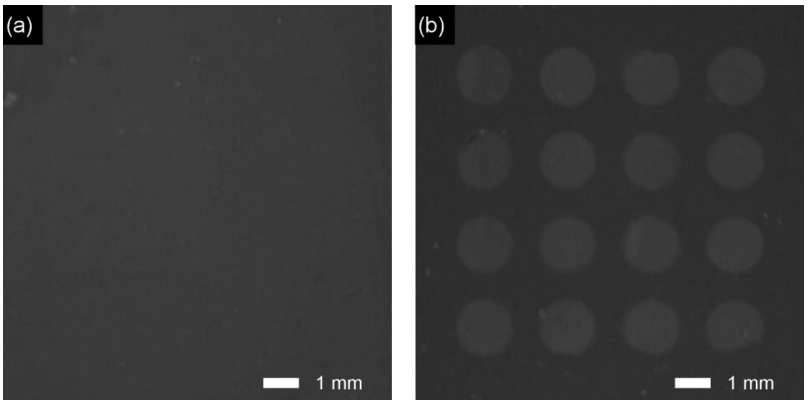

FIG. 3. Protection of the ECM from O_2 plasma oxidation with the physical mask. (a) Fluorescence image before oxidation. Note that the homogeneous fluorescence image indicates homogeneous immobilization of ECMs on the PDMS surface. (b) Fluorescence image after oxidation (output power: 100 W, oxidation time: 10 s).

bronectin under the physical mask remained on the surface after irradiatoxidation, and the pattern of the TRITC-fibronectin precisely corresponded to the array of cylindrical rods on the mask. These results indicate that the mask protected the ECM microarray from the O_2 plasma. In addition, the fact that the TRITC-labeled fibronectin unprotected by the mask was decomposed by O_2 plasma irradiatoxidation indicates that the spaces between the cylindrical rods on the physical mask and the ECM array layer were sufficiently oxidized by the O_2 plasma. Oxidation of the intervening spaces is important for sealing of the microfluidic network during the subsequent bonding of the microfluidic network layer and the ECM array layer.

The difference in fluorescence intensity between the protected TRITC-fibronectin after O_2 plasma oxidation and before oxidation was less than 12%. After oxidation, the physical mask was slightly deformed. These results indicate that the plasma made contact with the small open spaces between the mask and the surface and that the TRITC-labeled fibronectin in these spaces was damaged by the plasma. Perfect protection of the microarray might be accomplished by replacing the mask fabricated from PDMS, which is soft and damaged by O_2 plasma oxidation, with a mask made from a hard, highly resistant material such as polytetrafluoroethylene and by tightly clamping the physical mask and the ECM microarray in firm frames.

B. Fabrication of the perfusion culture microchamber array chip with ECMs

We fabricated the perfusion culture microchamber array chip with two types of ECMs on the bottoms of the microchambers by O_2 plasma bonding of the ECM array layer and the microfluidic network layer with physical masking. In a fluorescence micrograph of ECM spots on the ECM array layer [Fig. 4(a)], individual TRITC-labeled fibronectin spots (red) and fluorescein isothiocyanate-labeled collagen spots (green) (diameter: 1.4 mm; pitch: 2.25 mm) were visible, which indicates that the two different ECMs had been precisely immobilized on the PDMS plate [Fig. 4(a)]. A fluorescence micrograph of ECM spots on the bottom of the microchambers of the chip revealed that the shape and fluorescence intensity of the spots was not changed by the bonding process [Fig. 4(b)]. These results confirmed that the ECM microarray was sufficiently protected from the O_2 plasma by the mask.

During the introduction of gardenia blue solution, no leakage of the solution was observed, and it indicates that the microfluidic network layer and the ECM array layer were sufficiently sealed by means of O_2 plasma oxidation with masking [Fig. 4(c)]. The color densities in a bright field image of the microchamber array indicate the three depths (4.5, 52.5, and 271.2 μm) of the various microstructures of the microfluidic network. This image confirmed the fabrication of a multithickness microfluidic network by means of multilayer photolithography.

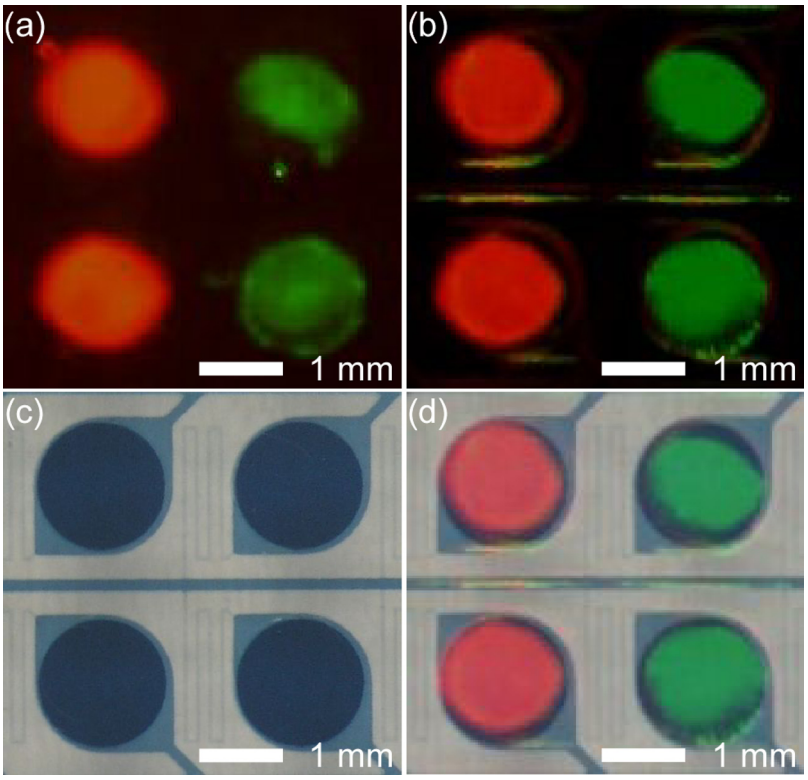

FIG. 4. Micrographs of the perfusion culture microchamber array chip. (a) Fluorescence micrograph of immobilized ECMs on the ECM array layer. (b) Fluorescence micrograph of ECM spots on the bottom of the microchambers of the chip. (c) Bright field image of the microchamber array. (d) Overlaid bright field and fluorescence micrographs of the microchamber array and immobilized ECMs.

An overlaid image of bright field and fluorescence micrographs of the microchamber array [Fig. 4(c)] and the ECM microarray [Fig. 4(b)] indicated that the ECM microarray was bonded to the microchamber array with an alignment difference of <100 μm [Fig. 4(d)]. The fabrication accuracy of the perfusion culture microchamber array with ECMs prepared by the present method is limited by the alignment difference, the resolution of the photolithography, and the diameter of the ECM spots. In the present study, we used printed photomasks with a 10 μm resolution for the multilayer photolithography. The diameter of the ECM spots is limited by the diameter of the through-hole in the stencil, which can be as small as 100 μm, as described in a previous report.[27] Therefore, the present method can be used to fabricate perfusion culture microchamber arrays with total dimension of the ECM diameter and the alignment difference as small as 300 μm. However, the homogeneity in the number of loaded cells in each microchamber is strongly affected by the dimensions and design of the microchamber array.[8] As the diameter of the microchamber is decreased, the distribution number of loaded cells in each microchamber decreases and the variability in the number of cells in each microchamber increases. The dimensions of the microchambers fabricated in this study (diameter: 1.53 mm, depth: 250 μm) were appropriate for perfusion culture in the microchamber array.

C. Cell culture in the perfusion culture microchamber array chip

A suspension of CHO-K1 cells was loaded into the perfusion culture microchamber array. During cell loading, no leakage of liquid from the microchip was observed, which indicates that the entire microfluidic network was sealed perfectly by means of O_2 plasma oxidation with masking. The loaded CHO-K1 cells adhered and extended in the fibronectin microchamber after static culture for 12 h [Fig. 5(a)], whereas cells did not extend to an unmodified PDMS micro-

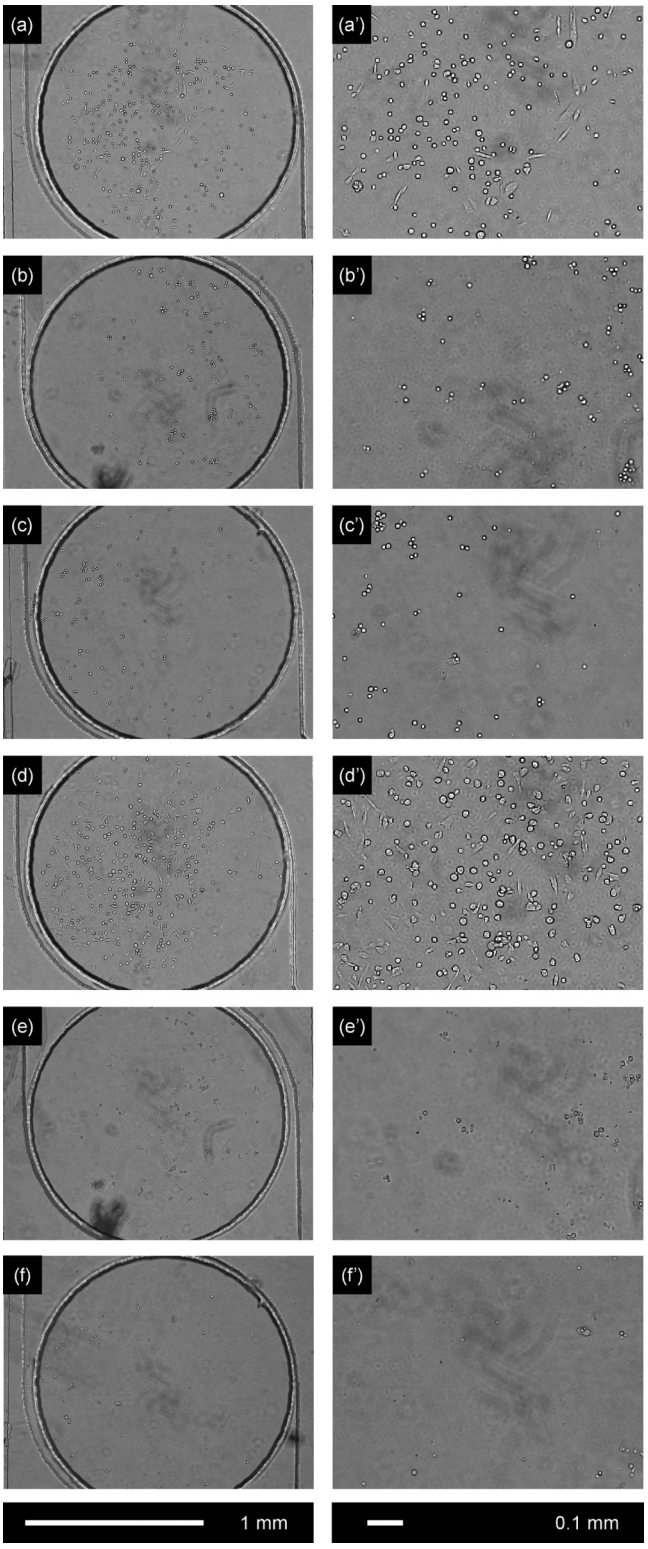

FIG. 5. Culture of CHO-K1 cells in the perfusion culture microchamber array chip. Micrographs of CHO-K1 cells after static culture for 12 h in (a) a fibronectin microchamber, (b) an unmodified PDMS microchamber, and (c) a collagen microchamber. Micrographs of CHO-K1 cells after perfusion culture for 1 d in (d) a fibronectin microchamber, (e) an unmodified PDMS microchamber, and (f) a collagen microchamber. (a′)–(f′) are enlargement in each microchamber.

chamber and the collagen microchamber after static culture for 12 h [Figs. 5(b) and 5(c)]. These results indicate that the immobilized fibronectin worked successfully for the cell adhesion.

Adhered CHO-K1 cells grew over the course of 1 d of perfusion culture in the fibronectin microchamber [Fig. 5(d)], whereas most of the cells were removed from the unmodified PDMS microchamber array and the collagen microchamber array after 1 d of perfusion culture [Figs. 5(e) and 5(f)]. The CHO-K1 cells adhered only weakly to the unmodified PDMS and the collagen, whereas adhesion to fibronectin was strong. These results, which are in agreement with the results of our previous study,[27] indicate that the cell-adhesive properties of ECMs were not changed by the plasma-bonding process. We also confirmed that the cell-adhesive properties of ECM could be maintained for at least 2 weeks in an incubator at 37 °C.

Generally, fibronectin is known to mediate cell adhesion through binding to integrins,[30] and fibronectin-coating of surface enhances the CHO cell adhesion.[31] Our result on fibronectin spot is in consistent with these previous studies. On the other hand, collagen is also known to enhance the adhesion of CHO cells.[32] Our result on collagen spot does not agree with the previous study, while the fluorescence from collagen spot was observed to a certain extent, as shown in Fig. 4. The contradiction is potentially explained by the further detailed study on the amount and conformation of the immobilized protein, and on the effect of the shear stress during the perfusion culture.

IV. CONCLUDING REMARKS

We described the fabrication of a perfusion culture microchamber array chip with ECMs by assembly of an ECM array layer and a microfluidic network layer by means of O_2 plasma oxidation and bonding with alignment. During O_2 plasma oxidation, the ECM microarray was protected from the plasma by a physical mask. The ECM microarray was precisely bonded to the microchamber array with an alignment difference of <100 μm. The array of immobilized fibronectin on the bottoms of the microchambers was effective for adhesion and growth of CHO-K1 cells. The perfusion culture microchamber array chip with ECMs is a unique closed system for cell culture and is expected to be useful for on-chip assays for various types of cells. In addition, the design of the chip allows for precise control of the cell culture environment by introduction of soluble factors through the microchannels, without interference by soluble factors secreted from neighboring spots.

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